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Abstract: Population kinetics of the agent of human granulocytic ehrlichiosis (aoHGE) were examined after needle and tickborne inoculation of C3H mice. Blood, skin, lung, spleen, liver, kidney, brain, lymph node, and bone marrow samples were analyzed by using real-time polymerase chain reaction (PCR) at various intervals after inoculation, using a p44 gene target. The highest number of copies of the p44 gene target occurred in blood and bone marrow samples, emphasizing aoHGE leukocytotropism. Numbers of copies of the p44 gene target in other tissues reflected vascular perfusion rather than replication. Needle-inoculated infected mice had earlier dissemination, but kinetics of infection in both groups were parallel, with declining rates of infection by day 20 and recovery in some mice on days 20-60 after inoculation. On the basis of an aoHGE lysate ELISA, mice seroconverted by day 10 after inoculation. Therefore, real-time PCR is useful for quantitative studies with the aoHGE in experimental infections, and results showed that needle inoculation can be used to study the aoHGE infection because of its similarity to tickborne inoculation

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Population kinetics of the agent of human granulocytic ehrlichiosis (aoHGE) were examined after needle and tickborne inoculation of C3H mice. Blood, skin, lung, spleen, liver, kidney, brain, lymph node, and bone marrow samples were analyzed by using real-time polymerase chain reaction (PCR) at various intervals after inoculation, using a *p44* gene target. The highest number of copies of the *p44* gene target occurred in blood and bone marrow samples, emphasizing aoHGE leukocytotropism. Numbers of copies of the *p44* gene target in other tissues reflected vascular perfusion rather than replication. Needle-inoculated infected mice had earlier dissemination, but kinetics of infection in both groups were parallel, with declining rates of infection by day 20 and recovery in some mice on days 20–60 after inoculation. On the basis of an aoHGE lysate ELISA, mice seroconverted by day 10 after inoculation. Therefore, real-time PCR is useful for quantitative studies with the aoHGE in experimental infections, and results showed that needle inoculation can be used to study the aoHGE infection because of its similarity to tickborne inoculation.

Human granulocytic ehrlichiosis (HGE) was recognized as a zoonotic disease in 1994 [1] and has been reported in regions of the United States [2, 3] and Europe [4, 5]. The agent of HGE (aoHGE) is transmitted from reservoir hosts to humans and/or susceptible animals primarily by ticks of the *Ixodes persulcatus* complex. HGE is manifested by fever, myalgia, leukopenia, and thrombocytopenia and can be fatal in some cases because of secondary complications [6, 7]. During the acute stage of infection, clusters of intracytoplasmic replicating organisms (morulae) are found in the cytoplasm of circulating granulocytes [8, 7]. The nucleotide sequence of the 16s RNA gene of the aoHGE has revealed high homology with 16s RNA genes of *Ehrlichia equi* and *E. phagocytophila* [9], which are agents associated with similar diseases in domestic animals, suggesting that they may be the same organism with minor genetic differences [10].

The pathogenesis of HGE can be studied, under laboratory conditions, with a number of animal models, such as horses [10], nonhuman primates [11], rabbits [12], dogs [13], and mice [14, 15]. Common methods of infecting laboratory animals include needle inoculation with cell culture–derived organisms or with blood from infected animals, as well as by allowing infected nymphal *Ixodes* ticks to feed on experimental hosts [15, 16, 17, 14]. Efficiency of transmission by both needle and tickborne inoculation is high and consistent, but the kinetics of infection in horses with the aoHGE indicate differences between these 2 methods of inoculation [17].

Perhaps the most utilitarian animal model for investigation of HGE pathogenesis is the inbred laboratory mouse, because of the armamentarium of investigative tools available for work with this species [14]. Because mice can be readily infected with the aoHGE by tickborne inoculation and because this route resembles natural infection, needle inoculation offers better consistency, control, and convenience for experimental studies. This study was undertaken to compare these 2 routes of inoculation and to determine the kinetics of infection and tissue distribution of the aoHGE during acute and chronic phases of infection after both routes of inoculation. To accomplish this, we used real-time polymerase chain reaction (PCR) for maximal sensitivity and quantitative analysis.

Materials and Methods

Mice. Three- to 5-week-old C3H/HeN (C3H) and C3H/Smn.CIcrHsd-*scid* (SCID) mice were purchased from Frederick Cancer Research Center and Harlan Sprague Dawley, respectively. Mice were maintained in a pathogen-free room in isolator cages and were provided with a commercial laboratory diet and water ad libitum.

aoHGE. The NCH-1 isolate of the aoHGE, a human isolate from Nantucket, Massachusetts [14], was maintained by serial

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SCID mouse passage, and the infection status was monitored by examining peripheral blood smears for morulae, as described elsewhere [15]. Mouse-passaged aoHGE was used for infection studies. In addition, the aoHGE was cultivated in HL-60 cell cultures for antigen (see ELISA below), as described elsewhere [15].

Infection of ticks. *I. scapularis* adults collected in New Haven, Connecticut, fed on uninfected rabbits until repletion and then were kept in a humidified chamber for oviposition. After hatching, larvae were tested and were sent to the University of California, Davis. Larvae were allowed to feed to repletion on aoHGE-infected C3H mice or uninfected mice (controls) and then were held at 21°C and >90% relative humidity until they molted into nymphs. C3H mice used for infestation were inoculated by intraperitoneal (ip) injection with blood obtained from aoHGE-infected and uninfected SCID mice (controls). Ten days after inoculation, each mouse was infested with >100 larvae. All ticks used in this study were derived from a single cohort of larvae. The percentage of infection and the number of organisms per tick were determined by testing 10% of the cohort of molted nymphs, using real-time PCR.

PCR. DNA used for PCR was extracted from ticks or from blood and tissue samples collected from mice at necropsy, using DNeasy tissue kits (Qiagen) according to the manufacturer's instructions. Before DNA extraction, all tissue samples were weighed, or aliquots from 50 μL of blood were obtained. For real-time quantitative PCR, 3 oligonucleotides (2 primers and a probe for the *p44* target gene; GenBank accession no. AF037599) were selected by means of Primer Express software (PE Biosystems). This target was selected because multiple *p44* genes encode 44-kDa major outer membrane proteins of the aoHGE that elicit dominant immune responses during early and late infection. Furthermore, immunization with these antigens partially protects mice from aoHGE challenge [18, 19]. Primers P44-802F (5′-ACGTCGAAAAAGGCGGAAA-3′) and P44-872R (5′-GCTACAGCTGCCGCGTTATC-3′) were synthesized to amplify a 71-bp fragment of a C-terminal conserved region of the HGE *p44* gene. The internal oligonucleotide probe P44-825P (5′-TGCAGGCACTCCGGAACCCGT-3′) was labeled at the 5′ end with the reporter dye 6-carboxy-fluorescein and at the 3′ end with the quencher dye 6-carboxy-tetramethyl-rhodamine. All reaction mixes were set up in an area isolated from PCR product analysis and sample preparation. DNA preparation, reaction assembly, and sample analysis were done in a dedicated area, using dedicated pipettors. Each PCR set-up included negative controls of no enzyme or a template control and an unrelated DNA control. Amplification, data acquisition, and data analysis were performed in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Quantification of the amount of HGE *p44* gene in samples was accomplished by using an absolute standard curve, to determine the starting number of copies. A Sequence Detection System software (version 1.6; Perkin Elmer) was used to analyze data, and results were exported to a Microsoft Excel worksheet for statistical analysis.

To quantify the numbers of copies of the HGE *p44* gene target in samples, a plasmid standard was prepared. Concentration of the plasmid with the insert was measured at a wavelength of $A°_{260}$ and was converted to the number of copies by using the molecular weight of DNA. To create a standard curve, plasmid DNA was used as template and was diluted in a 10^{10}-fold dilution series. Each plasmid DNA dilution representing the plot of the log of copy numbers versus values for the threshold cycle, which was defined using cycles 3–9 as baseline, was included in each PCR, which then was used to calculate an unknown amount of DNA in examined samples. The analytical sensitivity was 1–10^9 copies.

ELISA. Purified aoHGE was prepared with a discontinuous density Renografin gradient (Braccon Diagnostics), as described elsewhere [20]. To determine IgM and IgG titers, indirect ELISA was used. Microtiter plates (96-well; Nunc-Immuno MaxiSorp) were coated by adding, in alternate rows, purified aoHGE or uninfected HL-60 cell lysate at a concentration of 1 μg/mL in carbonate buffer (pH 9.6) and were incubated overnight at 4°C. Plates were washed and blocked with 1% bovine serum albumin. After washing, 100 μL of serial 2-fold dilutions of serum samples from experimentally infected or control mice (starting at 1:50) were added to each well and were incubated overnight at 4°C. The plates were washed again and then were incubated for 2 h with 100 μL of alkaline phosphatase–conjugated rat antibody to mouse IgG heavy and light chains (Jackson ImmunoResearch Laboratories) diluted 1:750. Plates were read on a dual-mode setting on an ELISA reader after color development (test wavelength, 405 nm; reference wavelength, 650 nm; Molecular Devices Corporation). Optimal concentrations of each substrate and timing were established before this experiment. Net absorbency value for each dilution of every serum sample was determined by subtracting HL-60 lysate values from their respective aoHGE values. Cut-off values were established on the basis of 3 SDs above the mean absorbance of normal mouse serum ($n = 15$). Titers were expressed as reciprocal serum dilutions.

Results

The experiment was designed to compare the kinetics of infection in C3H mice, after needle inoculation with blood from infected SCID mice, with the kinetics of infection after tick feeding. If the kinetics of infection by these routes of inoculation were sufficiently similar, the results would justify the use of needle inoculation for a more controlled approach in experimental studies.

Fifty-four ticks from the pool of 538 nymphal ticks used for inoculation were tested individually by PCR, to determine the percentage infection rate. Of the ticks tested, 37 (68.5%) were found to be aoHGE PCR-positive. Among ticks that were positive, the number of copies of the *p44* gene target per tick was as low as 10^3 and as high as 10^6 (mean ± SD, $8.34 \times 10^5 \pm 1.04 \times 10^5$ copies). Twenty-one of the 211 ticks in the uninfected tick pool were tested and were found to be negative.

Forty-eight C3H mice were inoculated by placing aoHGE-infected nymphal *I. scapularis* ticks on the dorsal cervical midline. Six infected nymphs were placed on each mouse and were allowed to feed to repletion. Twelve control mice were sham inoculated with uninfected ticks. Engorged nymphs were collected and individually tested by using PCR. Among 203 ticks from the infected group that were recovered after feeding to repletion, the *p44* gene target was detected in 121 ticks (59.6%) from the infected tick pool, indicating that each mouse was exposed to 2–6 infected ticks. In contrast, none of the 51 un-

Table 1. Agent of human granulocytic ehrlichiosis polymerase chain reaction (PCR) results from tissues of needle- and tick-inoculated infected mice, at various time intervals after inoculation.

Necropsy day,[a] route of inoculation	Skin	Blood	Prescapular lymph nodes	Mesenteric lymph nodes	Liver	Spleen	Kidneys	Lungs	Brain	Bone marrow	Total
3											
Needle	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4 (100)
Tick	2/8	3/8	5/8	1/8	1/8	3/8	2/8	4/8	3/8	2/8	5/8 (62.5)
5											
Needle	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4 (100)
Tick	6/8	6/8	6/8	5/8	4/8	6/8	4/8	6/8	4/8	5/8	6/8 (75)
10											
Needle	4/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4 (100)
Tick	2/8	2/8	2/8	2/8	2/8	2/8	2/8	2/8	0/8	2/8	2/8 (25)
20											
Needle	1/4	4/4	1/4	0/4	2/4	3/4	0/4	2/4	0/4	1/4	4/4 (100)
Tick	0/8	5/8	4/8	5/8	3/8	3/8	2/8	2/8	3/8	4/8	5/8 (62.5)
30											
Needle	2/4	3/4	1/4	1/4	3/4	3/4	1/4	1/4	1/4	2/4	3/4 (75)
Tick	0/8	3/8	5/8	2/8	1/8	3/8	2/8	2/8	2/8	2/8	5/8 (62.5)
60											
Needle	0/4	2/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4	2/4 (50)
Tick	0/8	1/8	0/8	1/8	2/8	0/8	0/8	1/8	0/8	2/8	2/8 (25)

NOTE. Data are no. of PCR-positive mice/no. of mice tested (%).
[a] Days after needle inoculation (days 5, 7, 12, 22, 32, and 62 after tick attachment, respectively).

infected engorged ticks collected from control mice was found to be positive.

For needle inoculation, mice were injected ip with infectious material, because previous studies have found that intradermal/subcutaneous inoculation requires a higher infectious dose [15]. It has been shown that ticks transmit the aoHGE 36–48 h after tick attachment [21, 20]. Therefore, to synchronize both experimental groups, needle inoculation of mice was done at 48 h after tick attachment in the other group. In addition, 24 C3H mice were inoculated ip with 0.1 mL of EDTA-anticoagulated blood pool from infected SCID mice (the percentage of granulocytes with morulae was adjusted to 10% by diluting blood with PBS). Six control mice were inoculated with serum from uninfected SCID mice. In an effort to approximate the aoHGE dose used in this experiment, similarly derived blood from infected SCID mice (same interval of infection, 10% morulae, 1 mL) was tested and found to contain 6×10^6 copies of the *p44* gene target.

On days 3, 5, 10, 20, 30, and 60 after inoculation (days 5, 7, 12, 22, 32, and 62 after tick attachment), 4 mice from the needle inoculation group and 8 mice from the tickborne inoculation group, as well as 1 mouse from the needle inoculation control group and 2 mice from the tickborne inoculation control group, underwent necropsy at each interval. Skin, lung, spleen, liver, kidney, brain, prescapular lymph nodes, mesenteric lymph nodes, bone marrow (femoral and tibial), and blood samples were collected for DNA extraction and PCR.

Infected mice were clinically normal, and the only discernable gross pathology among infected mice was splenomegaly. The mean spleen weight of control mice was 90 mg (SD, ±17.6 mg), whereas the mean spleen weights on days 10, 20, and 30 of needle-inoculated infected mice and of tick-inoculated infected mice were 205.9 mg (SD, ±32.7 mg) and 187.1 mg (SD, ±108 mg), respectively.

Infection was confirmed by PCR for all needle-inoculated infected mice on days 3–20, but ⩽75% of tick-inoculated infected mice were PCR positive at any interval (table 1). In contrast, all 10 tissue sites from all 18 control mice that were infected by needle and tickborne inoculation were PCR negative. Among infected mice, the *p44* gene target could be amplified from all tissues tested. Analysis of tissue distribution of the aoHGE revealed that the agent was distributed unevenly in tick-inoculated infected mice on days 3 and 5, but needle-inoculated infected mice had nearly universal infection of all tissues at these intervals. Comparison of the tissue distribution of aoHGE between the groups revealed no differences in distribution in mice examined

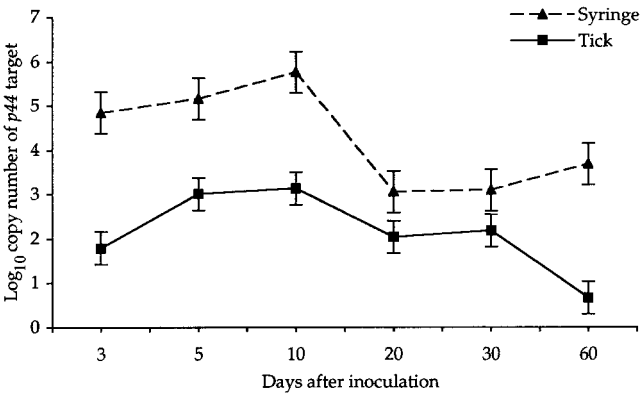

Figure 1. Mean nos. of copies of the agent of human granulocytic ehrlichiosis *p44* DNA (±SD) in blood from mice infected by needle or tickborne inoculation, at different time points after infection.

Table 2. Comparison of nos. of copies of the agent of human granulocytic ehrlichiosis (aoHGE) *p44* DNA extracted from different tissues of mice infected by needle and tickborne inoculation, at various time intervals after inoculation.

Days after needle inoculation,[a] route of inoculation	Skin[b]	Blood[c]	Prescapular lymph nodes	Mesenteric lymph nodes	Liver	Spleen	Kidney	Lung	Brain	Bone marrow[d]
3										
Needle	128 ± 117	121702 ± 88898	285 ± 317	184 ± 89	661 ± 592	3793 ± 2867	62 ± 41	295 ± 399	16 ± 9	822 ± 936
Tick	1.25 ± 1.5	636 ± 1010	177 ± 323	52 ± 104	236 ± 323	280 ± 560	12.5 ± 24.4	188 ± 353	8.2 ± 16	3100 ± 6200
5										
Needle	5.4 ± 6	145525 ± 64705	41 ± 18	117 ± 114	78 ± 72	748 ± 463	20 ± 10	38 ± 21	20 ± 12	199 ± 237
Tick	5.3 ± 5	1033 ± 967	318 ± 392	291 ± 158	1117 ± 1603	1620 ± 1596	166 ± 250	440 ± 547	10.4 ± 15.6	4423 ± 3652
10										
Needle	270 ± 266	574800 ± 590303	791 ± 1180	201 ± 128	1924 ± 2105	7805 ± 10960	325 ± 412	8715 ± 13930	42 ± 41	141 ± 187
Tick	478 ± 484	1373 ± 1240	607 ± 32	14 ± 6	5015 ± 1097	122 ± 56	743 ± 96	655 ± 339	0	37760 ± 15358
20										
Needle	4.3 ± 8.6	1139 ± 1132	3.56 ± 7.13	0	1.3 ± 2	5.7 ± 6.6	0	6.5 ± 12	0	4.9 ± 9.8
Tick	0	116 ± 54	29 ± 10	67 ± 58	133 ± 67	276 ± 192	10 ± 1	90 ± 27	4 ± 1	3130 ± 99
30										
Needle	4.7 ± 6.7	4827 ± 7685	3.8 ± 6.6	2.5 ± 4.3	5 ± 5	69 ± 98	2 ± 3.4	1.5 ± 2.5	2 ± 1	30 ± 31
Tick	0	151 ± 220	1216 ± 1983	190 ± 168	128 ± 221	289 ± 487	26 ± 27	76 ± 129	4.7 ± 5	3856 ± 3781
60										
Needle	0	2780 ± 3592	0	0	1.8 ± 2.5	0	0	0	0	54 ± 76
Tick	0	4.7 ± 6.6	0	4 ± 5.6	6.5 ± 2.6	0	0	3.5 ± 5	0	1251 ± 443

NOTE. Data are nos. of copies of aoHGE *p44* DNA, expressed as mean ± SD of positive samples.

[a] Days 5, 7, 12, 22, 32, and 62 after tick attachment, respectively.

[b] No. of copies of aoHGE *p44* DNA was expressed per microgram of tissue after adjusting the real-time polymerase chain reaction to the volume of the aliquot and the tissue weight.

[c] No. of copies in blood samples, as determined in 50 μL of purified blood.

[d] No. of copies of aoHGE *p44* DNA was expressed per microgram of bone marrow DNA.

on days 20, 30, and 60 after needle inoculation (days 22, 32, and 62 after tick attachment; table 2).

Quantitative analysis of the aoHGE *p44* gene target in various tissues at intervals after infection revealed no significant differences ($P > .05$, unpaired Student's *t* test) in skin, prescapular lymph nodes, mesenteric lymph nodes, spleen, kidneys, lungs, liver, and brain, and numbers of copies of the *p44* gene target in these organs tended to be low. In contrast, significantly higher numbers of copies of the *p44* gene were found in blood and bone marrow samples, but blood was clearly the predominant target organ for the aoHGE (figures 1 and 2). Mice infected by needle inoculation had significantly higher numbers of copies of the *p44* gene target in blood, compared with tick-inoculated infected mice, at days 3 and 5 ($P < .05$).

The kinetics of blood and bone marrow infection in mice infected with the aoHGE by needle inoculation versus those infected by tickborne inoculation were parallel, but needle-inoculated infected mice maintained consistently higher numbers of copies of the *p44* gene target in blood, and tick-inoculated infected mice maintained consistently higher numbers of copies of the organism in bone marrow (figures 1 and 2; $P < .05$, days 10 and 20). After day 10, numbers copies in infected mice declined in blood and bone marrow, as well as in all other tissues, in both groups (table 2; figures 1 and 2).

Seroconversion to aoHGE antigen, as detected by ELISA, occurred by day 10 in both groups. The mean ELISA titers in both groups of infected mice at any time point after inoculation were similar (figure 3). All mice infected with the aoHGE by needle inoculation seroconverted to the aoHGE antigen on days 10 and 20, and all aoHGE-inoculated infected mice that apparently had cleared infection, on the basis of PCR, by days 30 and 60 were seropositive. Thus, seroconversion accurately reflected infection. None of the PCR-negative tick-inoculated infected mice seroconverted to the aoHGE antigen.

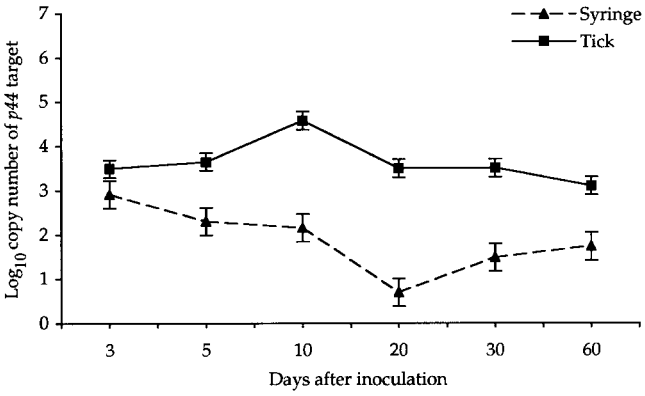

Figure 2. Mean nos. of copies of the agent of human granulocytic ehrlichiosis *p44* DNA (±SD) in bone marrow from mice infected by needle or tickborne inoculation, at different time points after inoculation.

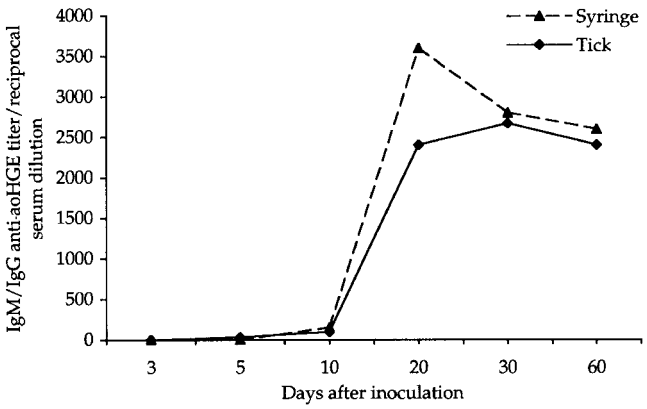

Figure 3. IgM/IgG ELISA antibody titers to the agent of human granulocytic ehrlichiosis (aoHGE) in polymerase chain reaction–positive mice, at various intervals after infection by needle and tick-borne inoculation.

Discussion

In a previous study [15], we were unable to detect the aoHGE in mice beyond 30 days after tickborne inoculation, using blood smears, HL-60 culture, and conventional PCR. Nevertheless, infection with the aoHGE was verified by xenodiagnosis at day 55 after inoculation. These observations emphasized the need to develop more-sensitive assays for the detection of the aoHGE in experimental studies. By using real-time PCR analysis, we were able to detect the aoHGE in both needle- and tick-inoculated infected mice at any time point from day 3 to day 60 after infection.

Real-time PCR offers not only sensitivity but also quantitative analysis of the kinetics of infection. By comparing needle inoculation with tickborne inoculation infection in various organs and at intervals $\leqslant$60 days after inoculation, we have shown that peripheral blood is the predominant target organ for the aoHGE. Involvement of a variety of tissues, such as skin, lymph nodes, kidneys, lungs, and brain, which were PCR positive but which had low numbers of target copies of the *p44* gene, suggests that the involvement of these organs is passive and probably a reflection of the blood within the vasculature of these sites. Involvement of the spleen and liver probably reflects, to a somewhat greater extent, the reticuloendothelial function of these organs, as well as the propensity of both organs to support extramedullary myelopoeisis in the adult mouse. The high rate of infection and relatively high numbers of copies of the aoHGE *p44* gene target in bone marrow is expected for an agent that replicates in hematopoeitic tissues. Thus, these results reinforce the notion that the aoHGE replicates in granulocytic and, occasionally, monocytic lineages [22, 23] without other host tissue targets.

Experimental studies with the aoHGE are fraught with significant difficulties in defining the infectious dose of the organism. This also was true in our study, since there was no way to equilibrate infectious dose between tickborne and needle inoculation. The biologic nature of the agent derived from these routes of inoculation is also likely to differ, since tickborne organisms are delivered to the host in an extracellular mode [14], whereas needle inoculation uses intracellular organisms. Furthermore, because the aoHGE replicates intracellularly, organisms cannot be quantified into infectious units in either ticks or blood samples, and numbers of copies of the gene target copy numbers may not necessarily reflect infectious units of organisms. Furthermore, our studies show that the numbers of copies of aoHGE organisms in ticks are highly variable and that not all mice become infected, even when 6 nymphal ticks were allowed to feed to repletion. We have shown elsewhere [20] that relatively large numbers (10^4–10^5) of the aoHGE are needed to infect mice. Others have suggested that large numbers of infected ticks are needed for successful transmission [17, 24]. On the other hand, one study demonstrated a very high rate of infection of mice after infestation with 5–8 infected nymphs [14]. These differences no doubt reflect variables in isolate, numbers within ticks, and numbers of ticks used for inoculation but underscore the problems that are consistent with tickborne infection in experimental studies.

Needle inoculation with the aoHGE is also less than ideal, because the organism is more efficiently transmitted by ip inoculation, compared with the putatively more natural route of cutaneous inoculation [15], and it is difficult to standardize infectious dose between studies. Nevertheless, needle inoculation offers standardization of dose within a given experiment and a more consistent rate of infection, as the current study demonstrated. This study was undertaken to determine whether needle and tickborne inoculation were of sufficient similarity that the more controlled, needle-inoculation method could be used with some degree of assurance that it mimicked natural infection. Results suggest that the kinetics of infection, target organs, and clearance patterns are basically similar, regardless of the inoculation method. The earlier and higher titers of aoHGE organisms in various tissues and the higher antibody titers to the aoHGE during active infection in needle-inoculated infected mice suggest that the mice received a higher infectious dose but that the basic kinetics of and response to infection in the 2 routes of inoculation were similar.

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